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(54) Peptide having inflammation affinity and radioactive diagnostic containing the same

Peptide mit Affinität für Entzündungen und deren radiomarkierte, diagnostische Zusammensetzungen

Peptides ayant une affinité pour des inflammations et leur composés diagnostiques radiomarqués

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Description

5 The present invention relates to a peptide having affinity with inflammatory cells and its derivatives or chemically modified substances. The present invention also relates to a radioactive diagnostic useful for diagnosing inflammation sites in living body of mammals including human, which comprises the above peptide and a radioactive metal label for the peptide.

10 The term "inflammation" covers so-called inflammatory responses wholly, including those caused by infectious diseases or on the periphery of tumors. Inflammation is induced by physical or chemical reactions due to heat, radiant energy, chemical substances, mechanical trauma or the like, and is also noted on infectious diseases caused by invasion into living bodies of foreign substances such as virus and bacteria, or on the periphery of tumors resulting from, for example, mutation of DNA. Inflammation is a series of reactions initiated by reaction of tissue cells subjected to inflammatory stimuli and by subsequent reactions in microcirculation system, particularly with protein permeation through blood vessels, followed by exudation and infiltration of leukocytes and granuloma formation to healing; it may be regarded as a biological defense reaction of the host for preventing foreign substance from invading into the living body or for normalizing inflammation sites.

15 Known radioactive diagnostic agents for imaging inflammations in living body including those caused by infectious diseases or on the periphery of tumors include gallium-67 citrate (Takashi Onishi, Nuclear Medicine (Kaku Igaku), Vol. 26, pp 1371-9, 1989), radioactive metal labeled polyclonal antibodies (JP-A-63502280 of Rabin. R. H et al, and Frans. H. M et al, Seminars in Nuclear Medicine, Vol 13, No. 2, April, pp 148 - 164, 1993), and radiolabeled leukocytes (Kazuo Ito, Nuclear Medicine (Kaku Igaku), Vol. 24, pp 341 - 51, 1987).

20 Gallium-67 citrate contains gallium-67 which has a long half-life of 3.26 days, and the radioactive metal labeled polyclonal antibodies per se are also long in half-life in blood; thus both agents make the radioactive metal stay in the body for a long period of time giving unnecessary exposure to the patient. Furthermore, imaging takes 20 hours or longer with both agents. Thus, it has been impossible to get diagnostic information quickly for patients in immediate 25 need of treatment.

25 Radiolabeled leukocyte diagnostic has been used in clinical practice as highly advanced medical care. However, this diagnostic requires a complicated procedure with advanced skill in which the surgeon has to prepare it by collecting blood from the patient, separating and refining leukocytes therefrom, labeling them with a radioactive metal such as indium-111 and again refining them prior to administration. This diagnostic is not prevalent because it requires a sterile room and other special equipment for preparation and thus is only usable in limited facilities In addition, the diagnostic 30 may be hazardous to the surgeon for infection if the patient is infected by viral hepatitis, HIV or the like. In the document WO-87/04351 there has been disclosed a method of detecting an inflammation site in an individual by administering to the individual a diagnostically effective amount of detectably labeled immunoglobulin or fragment thereof, wherein the immunoglobulin substantially accumulates at the site when the site is inflamed.

35 In the document EP-A-0 233 619 there has been disclosed a radioactive diagnostic or therapeutic agent, which comprises a metallic element-labeled high molecular compound, which comprises one unit of (1) a polyamine compound having at least three amino groups in the side chains per molecule, at least two units of (2) a chelate-forming compound having a carboxyl group, at least one unit of (3) a physiologically active substance and (4) at least two radioactive metallic elements, each unit of the chelate-forming compound (2) and the physiologically active substance 40 (3) being combined to any of the amino groups in the side chains of the polyamine compound (1) and each of the radioactive metallic element (4) being chelate-bonded to the chelate-forming compound (2).

45 In view of the above-mentioned disadvantages of conventional compounds and their radiolabelled products such as the unnecessary exposure to patients, limitation of facilities for preparation, difficulty to get image information quickly, complicated handling in preparation, and risk of infection to the operator, it is an object of the present invention to provide peptides and their chemically modified substances, radioactive metal labeled peptides derived therefrom, and radioactive diagnostics comprising such peptides, which are useful for imaging inflammation sites in living body of mammals including human, easy in preparation handling, and capable of accumulating at inflammation site quickly after administration and staying there for a suitable time for imaging while being excellent in clearance into urine.

50 As the result of the intensive researches made by the present inventors to attain the object, peptides at least a part of which comprises a specific amino acid sequence have been found to be useful and have led to accomplishment of the present invention. One aspect of the present invention is directed to peptides having affinity for inflammation sites, which consist of at least one amino acid sequence selected from the group consisting of

5
 LLGGPS,
 LLGGPSV,
 KEYKAKVSNKALPAPIEKTISK,
 KEYKCKVSNKALPAPIEKTISK,
 10 KTKPREQQYNSTYR, and
 KTKPREQQYNSTYRVV,

10 where A, C, E, G, I, K, L, N, P, Q, R, S, T, V, and Y represent amino acid residues expressed by standard one-letter symbols. These specific amino acid sequences hereinafter referred to as "basic amino acid sequences". Another aspect of the present invention is directed to derivatives of the above-mentioned peptides, the peptides and the derivatives labeled with radioactive metals, and radioactive diagnostics containing the peptides and/or the derivatives. Amino acid residues are expressed hereinafter by standard one- or three-letter symbols.

15 The peptides according to the present invention may be prepared by Fmoc method, a solid phase synthetic method, using a peptide synthesizer manufactured by Applied Biosystem. The target peptide may be obtained by simultaneous deprotection and separation from resinous carrier, of the completed peptide bonded to the solid layer, followed by purification with high-performance liquid chromatography (hereinafter referred to as "HPLC") utilizing a reverse phase column. The peptide may be synthesized in liquid phase or may be collected from animals or the like.

20 The derivatives of peptide having affinity for inflammation sites are those that are denatured or chemically modified so as to increase capability of accumulating at inflammatory cells, as explained hereunder. Examples of such derivatives include those in which peptides containing basic amino acid sequences are combined in parallel by use of Fmoc-K (Fmoc), those in which several peptides containing basic amino acid sequences are combined in tandem, those in 25 which peptides containing basic amino acid sequences are combined with a bifunctional cross linking agent, those in which peptides containing basic amino acid sequences are combined with a bifunctional cross linking agent and further be combined with a carrier such as polylysine or chitosan, those in which peptides are chemically modified by, for example, acetylation or amidation at N-terminal and/or C-terminal, and those in which peptides are substituted with amino acids of D-configuration in part or as a whole.

30 The above-mentioned bifunctional cross linking agent is useful for increasing peptide efficacy by combining a plurality of peptides having affinity for inflammation sites according to the present invention to increase peptide concentration or by combining with carriers to increase the amount of peptides to be held by the carrier. The said bifunctional cross linking agent is one capable of selectively bonding to amino acid residues, including sulfosuccinimidyl 4-(N-maleimidemethyl)cyclohexane-1-carboxylate (hereinafter abbreviated as "Sulfo-SMCC"), 3-maleimidebenzoic acid N-35 hydroxysuccinimide ester (hereinafter abbreviated as "MBS"), N-(ϵ -maleimidecaproyloxy)succinimide (hereinafter abbreviated as "EMCS"), and succinimidyl 4-(p-maleimidephenyl)butyrate (hereinafter abbreviated as "SMPB"). Particularly preferable one is Sulfo-SMCC.

35 Preferable examples of the carrier that can combine a plurality of the peptides having affinity with inflammation include polylysine and chitosan. Chitosan is particularly preferable.

40 A diagnostic useful for imaging inflammation can be obtained by labeling the present peptide having affinity for inflammation sites or its derivatives with radioactive metal ion such as technetium-99m (^{99m}Tc) and indium-111 (^{111}In). A bifunctional ligand is conveniently used for labeling with radioactive metal ion. Preferable examples of the ligand include diethylenetriamine pentaacetic acid (hereinafter abbreviated as "DTPA"), ethylenediaminetetraacetic acid (hereinafter abbreviated as "EDTA"), and 1,4,7,10-tetraazacyclododecane-1-aminoethylcarbamoylmethyl-4,7,10-tris[45 (R,S)-methylacetic acid] (hereinafter abbreviated as "DO3MA"). DTPA is most preferable.

45 Alternatively, labeling with a radioactive metal may be carried out by dissolving the present peptide or a derivative thereof in a physiological saline, an aqueous buffer solution or the like and allowing it to react with a radioactive metal. In case of technetium-99m, a peptide can be labeled by an ordinary process wherein a reduction agent having a proper redox potential such as stannous chloride is added to the peptide, followed by mixing with a sodium pertechnetate solution. In case of indium-111, a peptide can be labeled by mixing the peptide with a weak acidic aqueous solution containing indium-111 ions. If required, unreacted pertechnetate ions or indium-111 ions may be removed by HPLC or other means.

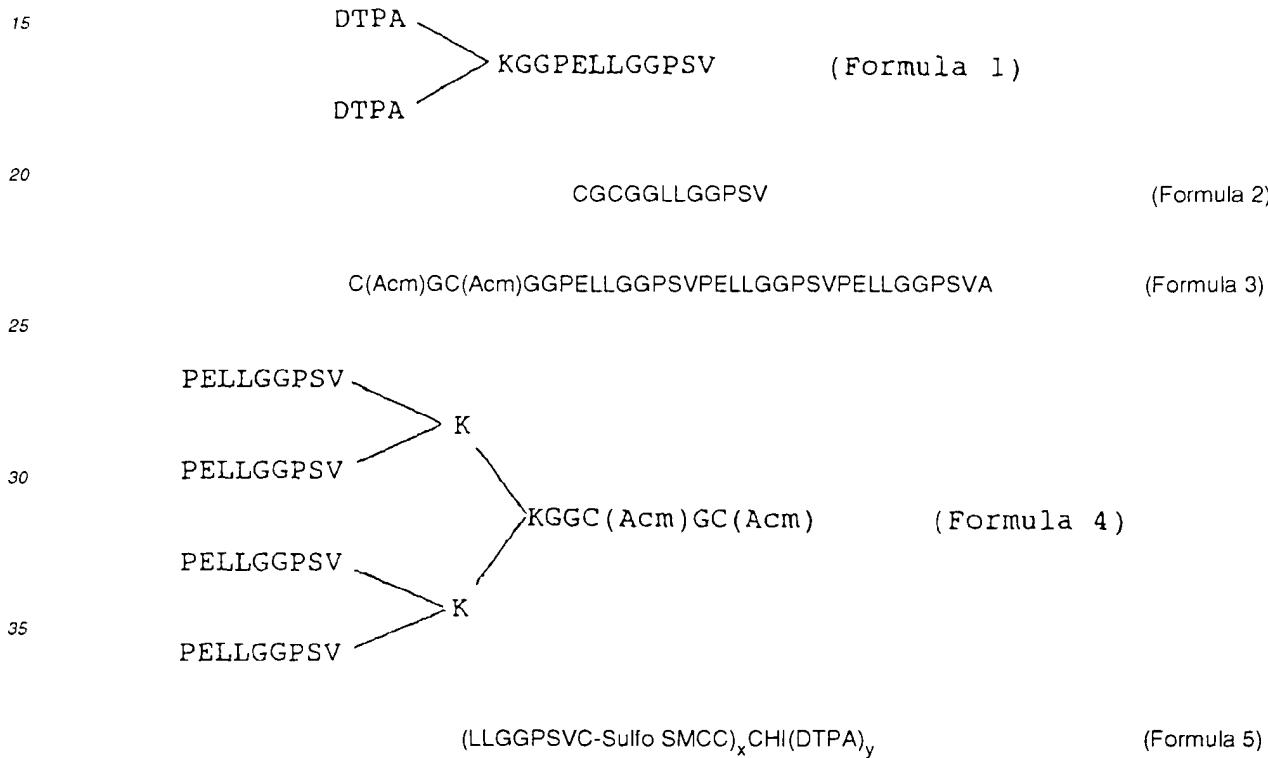
50 Furthermore, the present diagnostic may be provided in a form of a kit so that it can be prepared as required. The kit may include a pharmaceutically acceptable stabilizer such as ascorbic acid or p-aminobenzoic acid, a pH adjusting agent such as an aqueous buffer solution, an excipient such as D-mannitol, and an agent useful for improving radiochemical purity such as tartaric acid, or malonic acid.

55 Pharmaceutically acceptable peptide compounds comprising a peptide containing a radioactive metal in accordance with the present invention efficiently accumulate at inflammation sites just after generally-employed parenteral

administration such as intravenous bolus injection, attain quick distribution of the radioactive substance, provide a target site/background ratio sufficient to complete imaging within one hour after administration, and stay at the target site for a suitable period of time for imaging and thereafter are quickly excreted into urine via kidney; all of these features are desirable for a diagnostic. Thus, the peptide has excellent characteristics for overcoming conventional problems while enabling a diagnosis to be made by use of a radioactive imaging apparatus that is generally available.

The present radioactive diagnostic comprising the peptide having affinity for inflammation sites can be administered parenterally in accordance with a common practice such as intravenous bolus injection; the amount of administration is decided so as to obtain a radiation dose sufficient for imaging in consideration of various conditions such as weight and age of the patient as well as type of radioactive imaging apparatus. In case of human, a radiation dose from 185 to 1110 MBq is usually preferable.

Now, examples of the peptide having affinity for inflammation sites and its derivative according to the present invention are shown hereunder.



where $x + y \leq 5$

Now, the present invention is illustrated in more details by way of examples and the drawings in which

FIG. 1 is a whole body scintigram of an inflammation model rat one hour after administration of radioactive metal-labeled Peptide-4;

FIG. 2 is a whole body scintigram of an inflammation model rat one hour after administration of radioactive metal-labeled Peptide-5;

50 FIG. 3 is a whole body scintigram of an inflammation model rat one hour after administration of radioactive metal labeled Peptide-6; and

FIG. 4 is a whole body scintigram of a model rat with infectious disease one hour after administration of radioactive metal labeled Peptide-6.

55 While the technetium-99m labeling was made in the examples according to the process described in WO-A-

While the technetium-99m labeling was made in the examples according to the process described in WO-A-92/13572, the object of the example is to prove that the peptide according to the present invention is able to image

inflammation sites without change in performance regardless of kinds of nuclide or labeling method.

EXAMPLE 1

5 SYNTHESIS OF KGGPELLGGPSV (Peptide-1)

Synthesis was made with Peptide Synthesizer (Model 431A) manufactured by Applied Biosystems using HMP Resin by Fmoc process under 0.25 mM scale condition. Excision of the peptide was made by a reaction for 2.5 hours in aqueous 95 % trifluoroacetic acid (hereinafter abbreviated as "TFA") containing 2.5 % ethanedithiol (hereinafter 10 abbreviated as "EDT"). Purification was made by HPLC under the following conditions:

Column: YMC-PackR&D-ODS-5-ST (4.6 x 150 mm)

Elution velocity: 6 mL/min

Eluent A: 0.1 % TFA/purified water

15 Eluent B: 0.1 % TFA/acetonitrile

Concentration gradient: 0 min (10 % B) → 15 min (20 % B) → 40 min (50 % B)

Then, PICO-TAG-TM-Workstation manufactured by Waters was used for identifying the amino acid composition corresponding to the main peak. After it was confirmed that the composition was of the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 59.6 mg of the lyophilized product. The analytical 20 value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

Glu: 1.0 (1), Ser: 1.3 (1), Gly: 4.4 (4), Pro: 2.0 (2), Val: 1.1 (1), Leu: 2.1 (2), Lys: 1.2 (1).

For the purpose of confirming the reliability of the Peptide Synthesizer, the amino acid sequence of the product 25 peptide was determined by Protein Sequencer (a peptide automatic analyzer) Model 477A manufactured by Applied Biosystems. In addition, C-terminal analysis was made for confirming the C-terminal. As a result, the sequence from the N-terminal to S at the eleventh position coincided with the target sequence. The C-terminal analysis showed that the C-terminal was V. Thus, the peptide was confirmed to have an amino acid sequence of KGGPELLGGPSV.

30 EXAMPLE 2

SYNTHESIS OF CGCGGGLLGGPSV (Peptide-2)

Synthesis was made by the method described in EXAMPLE 1. Purification was made by HPLC under the following 35 conditions:

Column: YMC-PackR&D-ODS-5-ST (4.6 x 150 mm)

Elution velocity: 1 mL/min

Eluent A: 0.1 % TFA/purified water

40 Eluent B: 0.1 % TFA/acetonitrile

Concentration gradient: 0 min (5 % B) → 40 min (30 % B) → 120 min (60 % B)

After the amino acid composition corresponding to the main peak is determined in the same way as EXAMPLE 1 to confirm that the target peptide was obtained, the peak obtained was freeze-dried to yield 20 mg of the lyophilized 45 product. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

Ser: 1.0 (1), Gly: 4.9 (5), Pro: 1.0 (1), Val: 1.0 (1), Cys: 0.9 (2), Leu: 2.0 (2).

50 EXAMPLE 3

SYNTHESIS OF LLGGPSVC (Peptide-3)

A peptide having LLGGPSVC amino acid sequence was synthesized in an amount of 25 mg according to the method described in EXAMPLE 1, and the peptide was analyzed for the amino acid composition. The analytical value 55 (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

Ser: 0.9 (1), Gly: 2.0 (2), Val: 1.0 (1), Leu: 1.8 (2), Pro: 1.0 (1), Cys: 1.2 (1).

EXAMPLE 4SYNTHESIS OF C(Acm)GC(Acm)GGGKEYKAKVSNKALPAPIEKTIISK (Peptide-4)

5 Synthesis was made by the method described in EXAMPLE 1. Acm (acetamide methyl group) is a protective group for -SH group in cysteine. Excision of the peptide was made by allowing 100 mg of the resultant compound to react for 2.5 hours in 10 ml of aqueous solution in which 0.25 ml of EDT, 0.75 g of crystalline phenol, 0.5 ml of thioanisole, 0.5 ml of purified water and 9.5 ml of TFA were mixed. Purification was made by HPLC under the following conditions:

10 Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)
 Elution velocity: 8 mL/min
 Eluent A: 0.1 % TFA/purified water
 Eluent B: 0.1 % TFA/acetonitrile
 Concentration gradient: 0 min (10 % B) → 15 min (15 % B) → 40 min (50 % B)

15 After the amino acid composition corresponding to the main peak is determined in the same way as EXAMPLE 1 to confirm that the target peptide was obtained, the peak coinciding with the amino acid composition was freeze-dried to yield 45 mg of the lyophilized product with purity of 95 % or more. Since C(Acm) is unable to be identified by PICO-TAG method, the presence of C(Acm)GC(Acm) was confirmed by technetium-99m labeling. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

20 Asn:1.1 (1), Glu:2.0 (2), Ser: 2.3 (2), Gly:4.4 (4), Thr:1.0 (1), Ala:3.2 (3), Pro:2.1 (2), Tyr:0.6 (1), Val:0.9 (1), Ile:2.8 (2), Leu:1.3 (1), Lys:5.7 (6), Cys: - (2).

25 The amino acid sequence of the resulting peptide was determined using the same Sequencer as EXAMPLE 1; the sequence from the N-terminal to the 26th residues coincided with the target sequence. Thus, the peptide was confirmed to have an amino acid sequence of C(Acm)GC(Acm)GGGKEYKAKVSNKALPAPIEKTIISK.

EXAMPLE 5SYNTHESIS OF C(Acm)GC(Acm)GGKTKPQQYNSTYRVV (Peptide-5)

30 Synthesis was made by the method described in EXAMPLE 1. Excision of the peptide was made by allowing 150 mg of the resultant compound to react for 1.5 hours in 10 ml of aqueous solution in which 0.25 ml of EDT, 0.75 g of crystalline phenol, 0.5 ml of thioanisole, 0.5 ml of purified water and 9.5 ml of TFA were mixed. Purification was made by HPLC under the following conditions:

35 Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)
 Elution velocity: 8 mL/min
 Eluent A: 0.1 % TFA/purified water
 Eluent B: 0.1 % TFA/acetonitrile
 Concentration gradient: 0 min (10 % B) → 15 min (10 % B) → 90 min (40 % B)

40 Then, using the same analysis unit as EXAMPLE 1, the amino acid composition corresponding to the main peak was identified. After it was confirmed that the composition was of the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 51 mg of the lyophilized product of which purity was not less than 95 %. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

45 Asp:1.1 (1), Glx:3.1 (3), Ser:1.0 (1), Gly:3.3 (3), Arg:2.1 (2), Thr:2.2 (2), Pro:1.1 (1), Tyr:1.2 (2), Val:1.7 (2), Cys: - (2), Lys:5.9 (6)

EXAMPLE 6SYNTHESIS OF (PELLGGPSV) x 4 in parallel (K) x 2 in Parallel (KGKGC(Acm)GC(Acm)) (Peptide-6)

50 Synthesis was made by the method described in EXAMPLE 1. Purification was made by HPLC under the following conditions:

55 Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)

5 Elution velocity: 8 mL/min
 Eluent A: 0.1 % TFA/purified water
 Eluent B: 0.1 % TFA/acetonitrile
 Concentration gradient: 0 min (15 % B) → 15 min (15 % B) → 100 min (60 % B)

10 Then, using the same analysis unit as EXAMPLE 1, the amino acid composition corresponding to the main peak was identified. After it was confirmed that the composition was of the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 22.1 mg of the lyophilized product. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:
 15 Glu:3.9 (4), Ser:4.1 (4), Gly:11.8 (11), Pro:7.9 (8), Val:2.9 (4), Leu:7.6 (8), Lys:2.1 (3), Cys: - (2)

EXAMPLE 7

SYNTHESIS OF C(Acm)GC(Acm)GG[(PELLGGPSV) x 3 repeats in tandem]A (Peptide-7)

Synthesis was made by the method described in EXAMPLE 1. Purification was made by HPLC under the following conditions:

20 Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)
 Elution velocity: 8 mL/min
 Eluent A: 0.1 % TFA/purified water
 Eluent B: 0.1 % TFA/acetonitrile
 Concentration gradient: 0 min (15 % B) → 15 min (15 % B) → 100 min (60 % B)

25 Then, using the same analysis unit as EXAMPLE 1, the amino acid composition corresponding to the main peak was identified. After it was confirmed that the composition was of the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 55.6 mg of the lyophilized product. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:
 30 Glu:3.1 (3), Ser:3.0 (3), Gly:9.7 (9), Pro:6.9 (2), Val:2.9 (3), Leu:6.0 (6), Ala:2.0 (1)

EXAMPLE 8

INTRODUCTION OF BIFUNCTIONAL LIGAND (DTPA) INTO LYSINE RESIDUE OF Peptide-1

Peptide-1 obtained in EXAMPLE 1, in an amount of 5.0 μ mol, was dissolved in 3.0 ml of a 0.1 M phosphate buffer solution (pH 8.0); 10 times volume of DTPA anhydride was added to the mixture with agitation at room temperature and allowed to react for 30 minutes. Three peaks were obtained from the mixture by HPLC separation using 230 nm detection wavelength. Indium-111 was labeled to each of the peak components for determining the target peak; the labeling ratio was determined by electrophoresis using acetylcellulose membrane.

40 Radiochemical purity was 96.0 % for peak 1, 98.0 % for peak 2, and 38.0 % for peak 3. The high labeling ratio of 98.0 % for the peak 2 component led to the conclusion that this component was the target compound having the bifunctional ligand combined to the peptide. The peak 2 component was freeze-dried to yield 7.1 mg of the DTPA combined peptide.

EXAMPLE 9

INTRODUCTION OF BIFUNCTIONAL LIGAND (DTPA) INTO CHITOSAN-PENTAMER (CHI-PENTAMER)

50 Chitosan-pentamer, in an amount of 32.8 μ mol, was dissolved in 3.0 ml of a 0.1 M bicarbonate buffer solution (pH 9.7); 5 times volume of DTPA anhydride was added to the mixture with agitation at room temperature and allowed to react for 1 hour. The mixture solution was refined by electrodialysis for 2.5 hours using the unit (Micro Acilyzer S1) of Asahi Chemical Industry Co., Ltd. The resultant sample was analyzed quantitatively by ninhydrine reaction of the unreacted primary amines. The analysis showed the presence of at least two unreacted primary amines, indicating from 1 to 3 DTPAs were introduced into the CHI-pentamer.

EXAMPLE 10SYNTHESIS OF Peptide-3-Sulfo-SMCC-CHI-pentamer-DTPA

5 DTPA-CHI-pentamer obtained in EXAMPLE 9, in an amount of 10 nmol, was dissolved in 8.0 ml of a 50 mM borate buffer solution (pH 7.6); 50 nmol of Sulfo-SMCC was added to the aqueous solution with agitation at 30 °C and the mixture was allowed to react for 1 hour. Purification was made by electrodialysis using the unit of Asahi Chemical Industry Co., Ltd. followed by dry-freezing. A 37.5 % portion of the product compound was aliquoted and dissolved into a 0.1 M tris-HCl buffer solution (pH 7.0). To this mixture, 12 µmol of Peptide-3 obtained in EXAMPLE 3 was added; 10 the whole was allowed to react in a low temperature room overnight, then concentrated and purified by HPLC. Two peaks were obtained using 215 nm detection wavelength (retention time: 61 minutes for peak 1, and 73 minutes for peak 2). Indium-111 was labeled to each of the peak components, and radiochemical purity was determined by electrophoresis; peak 1 was 82.6 % and peak 2 was 83.4 %. Since hydrophobicity of the whole compound increases as 15 the number of the peptides combined to CHI increases, further experiments were made using the peak 2 that appeared to be the target compound because of the longer HPLC retention time.

EXAMPLE 11N-TERMINAL ACETYLATION OF KGGPELLGGPSV (Peptide-1)

20 An amount of 8.0 µmol of Peptide-1 prepared in EXAMPLE 1 was dissolved in 1.0 mL of a 0.3 M phosphate buffer solution (pH 7.3); 20 µl of acetic anhydride was added thereto with agitation at 4 °C and the mixture was allowed to react for 12 hours. After the completion of reaction, the N-terminal acetylation was checked by measuring absorbance at a wavelength of 570 nm. Result was 1.734 for N-terminal amine and 0.249 for N-terminal acetylated, which means 25 that 85.7 % was acetylated. It is known that as combination of acetyl group to N-terminal increases, the hydrophobicity of the peptide and retention time are extended upon reverse-phase HPLC. On this basis, the HPLC analysis was conducted and indicated that the retention time was 13.72 minutes for the starting peptide-1, and 14.93 minutes for the N-terminal acetylated peptide-1. The extension of about one minute of the retention time verified the acetylation 30 of the N-terminal of the peptide.

EXAMPLE 12C-TERMINAL AMIDATION AND N-TERMINAL ACETYLATION OF C(Acm)GC(Acm)
GGGKEYKAKVSNKALPAPIEKTSK (Peptide-4)

35 In the process described in EXAMPLE 1, PAL (Peptide Amide Linker) Resin made by MILLIPORE was used in place of the HMP Resin to synthesize C(Acm)GC(Acm)GGGKEYKAKVSNKALPAPIEKTSK of which C-terminal was amidated. Then, its N-terminal was acetylated by acetic anhydride using N-hydroxybenzotriazole (HOBT) as an activation agent.

40 Excision of the peptide was made by allowing 150 mg of the resultant compound to react for 2 hours in 10 ml of aqueous solution in which 0.25 ml of EDT, 0.75 g of crystalline phenol, 0.5 ml of thioanisole, 0.5 ml of purified water and 9.5 ml of TFA were mixed. Purification was made by HPLC under the following conditions:

45 Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)
Elution velocity: 8 mL/min
Eluent A: 0.1 % TFA/purified water
Eluent B: 0.1 % TFA/acetonitrile
Concentration gradient: 0 min (10 % B) → 15 min (15 % B) → 75 min (50 % B)

50 Then, using the same analysis unit as EXAMPLE 1 for analyzing the amino acid composition, the amino acid composition corresponding to the resultant main peak was identified. After it was confirmed that the composition was of the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 32 mg of the lyophilized product of which purity was not less than 95 %. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

55 Asn:1.1 (1), Glu:2.1 (2), Ser:2.0 (2), Gly:3.8 (4), Thr:1.1 (1), Ala:3.1 (3), Pro:2.1 (2), Tyr:0.6 (1), Val:1.0 (1), Cys: - (2), Ile:2.2 (2), Leu:1.2 (1), Lys:5.9 (6)

EXAMPLE 13C-TERMINAL AMIDATION AND N-TERMINAL ACETYLATION OF C(Acm)GC(Acm)GGKTKPREQQYNSTYRVV
(Peptide-5)

5 In the process described in EXAMPLE 1, PAL Resin manufactured by MILLIPORE was used in place of the HMP Resin to synthesize C(Acm)GC(Acm)GGKTKPREQQYNSTYRVV of which C-terminal was amidated. Then, its N-terminal was acetylated by acetic anhydride using N-hydroxybenzotriazole (HOBr) as an activation agent.

10 Excision of the peptide was made by allowing 150 mg of the resultant compound to react for 1.5 hours in 10 ml of aqueous solution in which 0.25 ml of EDT, 0.75 g of crystalline phenol, 0.5 ml of thioanisole, 0.5 ml of purified water and 9.5 ml of TFA were mixed. Purification was made by HPLC under the following conditions:

Column: YMC-PackR&D-ODS-5-ST (20 x 150 mm)

Elution velocity: 8 mL/min

15 Eluent A: 0.1 % TFA/purified water

Eluent B: 0.1 % TFA/acetonitrile

Concentration gradient: 0 min (10 % B) → 15 min (10 % B) → 75 min (50 % B)

20 Then, using the same analysis unit as EXAMPLE 1 for analyzing the amino acid composition, the amino acid composition corresponding to the resultant main peak was identified. After it was confirmed that the composition was the target peptide, the peak coinciding with the amino acid composition was freeze-dried to yield 23 mg of the lyophilized product of which purity is not less than 95 %. The analytical value (number per molecule) for the amino acid composition of the product peptide is shown below together with the theoretical value in parentheses:

25 Asp:1.1 (1), Glx:3.2 (3), Ser:0.9 (1), Gly:3.1 (3), Arg:2.2 (2), Thr:2.2 (2), Pro:1.1 (1), Tyr:1.3 (2), Val:1.6 (2), Cys: - (2), Lys:5.9 (6)

EXAMPLE 14PREPARATION OF INDIUM-111 LABELED PEPTIDE

30 Peptide-1-DTPA obtained in EXAMPLE 8, in an amount of 100 through 200 nmol was adjusted to pH 5.7 in a 0.1 M citrate buffer solution, and then 37 to 74 MBq of indium (¹¹¹In) chloride was added thereto. After agitation, the solution was left as such for 15 minutes. A part of the solution was taken and checked for the labeling percentage by electrophoresis. The radiochemical purity of the target compound was 98 %. Peptide-3-Sulfo-SMCC-CHI-DTPA obtained in EXAMPLE 10 was also labeled with In-111 in the same way as above, and the radiochemical purity of the target compound was 90 %.

EXAMPLE 15TECHNETIUM-99m LABELING OF PEPTIDES CONTAINING CGC SEQUENCE

40 An amount of 240 - 300 nmol of Peptide-2 obtained in EXAMPLE 2 was taken into each vial, and a volume of a 0.1 M phosphate buffer solution (pH 8.0) was added thereto to adjust the whole molarity to 300 μmol. Atmosphere in the vial was replaced with argon; then 180 - 300 nmol of dithiothreitol was added thereto. The mixture was then allowed to react for one hour at room temperature. Next, 120 nmol of stannous chloride and 740 - 1110 MBq of sodium pertechnetate were added. The mixture was left for one hour under gentle agitation.

45 Peptide-2 labeled with technetium-99m was yielded. Peptide labeled had radiochemical purity not less than 90 %.

EXAMPLE 16PREPARATION OF GLUCOHEPTANATE LABELED WITH TECHNETIUM-99m

50 An amount of 20.4 μmol of glucoheptanic acid was dissolved into a 0.1 M phosphate buffer solution (pH 8.0) to adjust the total volume to 450 μl. Atmosphere in the vial was replaced with argon; then 120 μmol of stannous chloride and 1.5 - 2.2 GBq of sodium pertechnetate were added. The mixture was gently agitated for 30 minutes. Thereby, glucoheptanate labeled with technetium-99m was yielded.

EXAMPLE 17TECHNETIUM-99m LABELING OF PEPTIDES CONTAINING C(Acm)GC(Acm) SEQUENCE

5 Experiments were made for all the peptides those prepared in EXAMPLES 4, 5, 6 and 7. Each of the peptides was taken, in an amount of 0.2 - 1.0 μ mol into a vial respectively, and a volume of a 0.1 M phosphate buffer solution (pH 8.0) was added thereto to adjust the whole volume to 500 μ l. Atmosphere in the respective vial was replaced with argon. To each vial, 500 μ l of 1.5 - 2.2 GBq/ml glucoheptanate labeled with technetium-99m yielded in EXAMPLE 16 was added and quickly agitated. Thereafter, the mixture was allowed to react for 20 minutes in a boiling water bath.

10 Labeling percentage of all the experimented peptides after cooling was 90 - 95 % according to TLC.

EXAMPLE 18IMAGING OF INFLAMMATION SITES USING INDIUM-111 LABELED PEPTIDE

15 To the right femoral part of Sparague-Davley Rats weighing about 220 g, 100 μ l of turpentine oil was subcutaneously administered. After 24 hours, when inflammation was clearly observed, Ravonal anesthesia was applied; then, Peptide-1-DTPA-indium-111 and Peptide-3-Sulfo-SMCC-CBI-DTPA-indium-111 obtained in EXAMPLE 14 were respectively administered in an amount of 3.7 MBq - 7.4 MBq to the respective tail vein. Images were obtained with gamma camera one, three, and six hours later. A site of interest was set on the image, where the ratio ([T]/[B] ratio) of total counts for the inflammation site [T] to total counts for the corresponding normal site of the opposite leg [B] was determined. The [T]/[B] ratio, one hour after the administration, was 2.63 for Peptide-1-DTPA-indium-111 and 2.09 for Peptide-3-DTPA-indium-111, whereby the focal site was evidently imaged. Table 1 shows time course of the [T]/[B] ratios (mean value \pm standard error) for three rats with respective peptides.

25

Table 1

[T]/[B] ratio of In-111 labeled peptides in inflammation model rats				
Labeled Peptide	Period of time after administration			
	1 hr.	3 hrs.	6 hrs.	
Peptide-1	2.63 \pm 0.19	2.30 \pm 0.13	1.71 \pm 0.18	
Peptide-3	2.09 \pm 0.18	2.46 \pm 0.29	2.78 \pm 0.15	

35 EXAMPLE 19IMAGING OF INFLAMMATION SITES USING TECHNETIUM-99m LABELED PEPTIDE

40 After Ravonal anesthesia was applied to the model rats described in EXAMPLE 18, 37 - 74 MBq of technetium-99m labeled Peptide-2 obtained in EXAMPLE 15, Peptide-4, Peptide-5, Peptide-6 and Peptide-7 obtained in EXAMPLE 17 were each administered to the respective tail vein. Images were obtained with gamma camera 30 minutes and one, three, and six hours later respectively. A site of interest was set on the image and the [T]/[B] ratio was determined. The [T]/[B] ratio, one hour after the administration, was 3.35 for Peptide-2, 4.98 for Peptide-6, 3.36 for Peptide-7, 4.27 for Peptide-4 and 4.41 for Peptide-5 whereby the focal site was evidently imaged. Table 2 shows time course of the [T]/[B] ratios (mean value \pm standard error) for three rats with respective peptides.

45

Table 2

[T]/[B] ratio of Tc-99m labeled peptide in inflammatory model rats				
Labeled Peptide	Period of time after administration			
	30 mins	1 hr.	3 hrs.	6 hrs.
Peptide-2	-	3.35 \pm 0.33	2.78 \pm 0.23	2.82 \pm 0.17
Peptide-4	2.47 \pm 0.17	4.27 \pm 0.26	3.10 \pm 0.20	-
Peptide-5	2.61 \pm 0.08	4.41 \pm 0.62	3.85 \pm 0.63	-
Peptide-6	-	4.98 \pm 0.23	4.61 \pm 0.49	3.20 \pm 0.39
Peptide-7	-	3.36 \pm 0.31	2.18 \pm 0.39	2.26 \pm 0.20

EXAMPLE 20IMAGING OF RAT MODEL WITH INFECTIOUS DISEASE USING TECHNETIUM-99m LABELED PEPTIDE

5 In 1.0 mL of physiological saline, 10^8 of viable microbe cells of *Staphylococcus aureus* were suspended. An aliquot of 100 μ l was administered intramuscularly to the right femoral region of Sparague-Davely Rats weighing about 220 g. After 24 hours, Ravonal anesthesia was applied to the model rats that had clearly evident inflammation; then, 37 - 74 MBq of technetium-99m labeled Peptide-6 obtained in EXAMPLE 8 was administered to the tail vein. Images were obtained with gamma camera one, three, and six hours later respectively. A site of interest was set on the image, and the [T]/[B] ratio was determined. One hour after the administration, the [T]/[B] ratio showed 1.92 indicating that focal region was evidently imaged. Table 3 shows time course of the [T]/[B] ratios (mean value \pm standard error) with this peptide.

Table 3

[T]/[B] ratio of Tc-99m labeled peptide in model rats with infectious disease			
Labeled Peptide	Period of time after administration		
	1 hr.	3 hrs.	6 hrs.
Peptide-6	1.92 ± 0.10	1.58 ± 0.05	1.52 ± 0.17

EXAMPLE 21SAFETY OF THE PEPTIDES

25 Assuming that clinical dosage to human is 1.0 mg/60 kg, bolus administration of Peptide-1, Peptide 4 and Peptide-5 in an amount of 8.3 μ g per 1.0 g of rat and physiological saline as control were made respectively to the tail vein of the rat. Immediately after the administration, behavior of the rat was observed. The observation was continued until five days later; zero minute, ten minutes, three hours, six hours, one day, two days, three days, four days, and five days later. Just after the peptide administration, symptoms such as slobber, vomiting, ocular proptosis, and behavioral abnormality were not observed. Furthermore, extreme change in weight, i.e., weight gain or loss was not observed until five days later. After weighing five days later, the rats were dissected. Abnormality in tissue was checked with the naked eye; abnormality in any tissue was not noted such as hemostatis, pigmentation, or discoloration. These gave estimation that more than 1,000 times administration of the assumed clinical dosage would be safe.

35 As explained, the present invention provides a peptide and its chemically modified substances, radioactive metal labeled peptides derived therefrom, and radioactive diagnostics comprising such peptide, which are useful for imaging inflammation sites in living body of mammals including human and easy in preparation handling, and accumulate at inflammation sites immediately after the administration and stay there for a time suitable for imaging while being excellent in clearance via kidney into urine; thereby eliminating unnecessary exposure to patients, limitation of facilities for preparation, difficulty to get image information quickly, complicated handling and skill in preparation, and risk of infection to the operator. According to the present invention, the imaging is possible in several ten minutes after administration.

45 **Claims**

1. A peptide having affinity for inflammation sites, which consists of at least one of the following amino acid sequences:

50 **LLGGPS,**
LLGGPSV,
KEYKAKVSNKALPAPIEKTISK,
KEYKCKVSNKALPAPIEKTISK,
55 **KTKPREQQYNSTYR,**
KTKPREQQYNSTYRVV,

wherein A, C, E, G, I, K, L, N, P, Q, R, S, T, V, and Y represent amino acid residues expressed by standard one-letter symbols.

2. A peptide derivative having affinity for inflammation sites, which consists of the peptide of Claim 1 combined with a bifunctional cross linking agent.
3. A peptide derivative having affinity for inflammation sites according to claim 1, which consists of the peptide of claim 1 combined with a bifunctional ligand, wherein the bifunctional ligand is one selected from the group consisting of diethylenetriamine pentaacetic acid, ethylenediaminetetraacetic acid, and 1,4,7,10-tetraazacyclododecane-1-aminoethylcarbamoylmethyl-4,7,10-tris[(R,S)-methylacetic acid].
4. A peptide derivative having affinity for inflammation sites, which consists of the peptide of claim 2 combined with a carrier through the bifunctional cross linking agent.
5. A peptide derivative having affinity for inflammation sites according to claim 4, in which a bifunctional ligand is combined with the carrier, which bifunctional ligand is one selected from the group consisting of diethylenetriamine pentaacetic acid, ethylenediaminetetraacetic acid, and 1,4,7,10-tetraazacyclododecane-1-aminoethylcarbamoylmethyl-4,7,10-tris[(R,S)-methylacetic acid].
6. A peptide derivative having affinity for inflammation sites according to Claim 2, 4 or 5, wherein the bifunctional cross linking agent is one selected from the group consisting of sulfosuccinimidyl 4-(N-maleimidemethyl) cyclohexane-1-carboxylate, 3-maleimidobenzoic acid N-hydroxysuccinimide ester, N-(ϵ -maleimidecaproxy) succinimide, and succinimidyl 4-(p-maleimidephenyl) butyrate.
7. A peptide derivative having affinity for inflammation sites according to claim 4 or 5, wherein the carrier is polylysine or chitosan.
8. A peptide derivative labeled with a radioactive metal, which consists of the peptide derivative of Claim 3 or 5, with which a radioactive metal ion is coordinated.
9. A peptide derivative labeled with a radioactive metal according to claim 8, wherein the radioactive metal ion is technetium-99m or indium-111.
10. A radioactive diagnostic comprising the radioactive metal labeled peptide derivative of claim 8 or 9.

Patentansprüche

1. Ein Peptid mit einer Affinität für entzündete Stellen, welches Peptid aus mindestens einer der nachfolgend wiedergegebenen Aminosäuresequenzen besteht:

LLGGPS,
LLGGPSV,
KEYKAKVSNKALPAPIEKTISK,
KEYKCKVSNKALPAPIEKTISK,
KTKPREQQYNSTYR,
KTKPREQQYNSTYRVV,

im Rahmen welcher vorstehend genannten Aminosäuresequenzen die Buchstaben A, C, E, G, I, K, L, N, P, Q, R, S, T, V und Y jeweils die entsprechenden, mit Hilfe der standardmäßig verwendeten Einbuchstabsymbole wiedergegebenen Aminosäurereste darstellen.

2. Ein Peptidderivat mit einer Affinität für entzündete Stellen, welches Peptidderivat aus dem Peptid nach Anspruch 1 besteht, kombiniert mit einem bifunktionellen Vernetzer.

3. Ein Peptidderivat mit einer Affinität für entzündete Stellen entsprechend dem vorstehenden Anspruch 1, welches Peptidderivat aus dem Peptid nach Anspruch 1 besteht, kombiniert mit einem bifunktionellen Liganden, welcher bifunktionelle Ligand aus der Gruppe ausgewählt wird, die aus den Verbindungen Diethylentriaminpentaessigsäure, Ethyldendiamintetraessigsäure und 1,4,7,10-Tetraazacyclododecan-1-aminoethylcarbamoylmethyl-4,7,10-tris[(R,S)-methylessigsäure] besteht.

5

4. Ein Peptidderivat mit einer Affinität für entzündete Stellen, welches Peptidderivat aus dem Peptid nach Anspruch 2 besteht und zudem mit Hilfe des besagten, bifunktionellen Vernetzers außerdem auch noch mit einem Trägermaterial kombiniert ist.

10

5. Ein Peptidderivat mit einer Affinität für entzündete Stellen entsprechend dem vorstehenden Anspruch 4, im Rahmen welchen besagten Peptidderivates ein bifunktioneller Ligand mit dem besagten Trägermaterial kombiniert ist, welcher bifunktionelle Ligand aus der Gruppe ausgewählt wird, die aus den Verbindungen Diethylentriaminpentaessigsäure, Ethyldendiamintetraessigsäure und 1,4,7,10-Tetraazacyclododecan-1-aminoethylcarbamoylmethyl-4,7,10-tris[(R,S)-methylessigsäure] besteht.

15

6. Ein Peptidderivat mit einer Affinität für entzündete Stellen entsprechend einem der vorstehenden Ansprüche 2, 4 oder 5, im Rahmen welchen besagten Peptidderivates der bifunktionelle Vernetzer eine Verbindung darstellt, die aus der Gruppe ausgewählt wird, welche aus den Verbindungen Sulfosuccin-imidyl-4-(N-maleimidmethyl)cyclohexan-1-carboxylat, 3-Maleimidbenzoësäure-N-hydroxysuccinimidester, N-(ϵ -Maleimidcaproyloxy)succinimid und Succinimidyl-4-(p-maleimidphenyl)-butyrat besteht.

20

7. Ein Peptidderivat mit einer Affinität für entzündete Stellen entsprechend einem der vorstehenden Ansprüche 4 oder 5, im Rahmen welchen besagten Peptidderivates das besagte Trägermaterial Polylysin oder Chitosan ist.

25

8. Ein mit einem radioaktiven, metallischen Element markiertes Peptidderivat, welches besagte Peptidderivat aus dem Peptidderivat nach einem der vorstehenden Anprüche 3 oder 5 besteht und an welches letztgenannte Peptidderivat ein radioaktives Metallion koordinativ gebunden ist.

30

9. Ein mit einem radioaktiven, metallischen Element markiertes Peptidderivat nach dem vorstehenden Anspruch 8, im Rahmen welchen besagten Peptidderivates das besagte, radioaktive Metallion entweder Technetium-99m oder Indium-111 ist.

35

10. Eine radioaktiv markierte, diagnostisch wirksame Zusammensetzung, welche ein mit Hilfe eines radioaktiven, metallischen Elements markiertes Peptidderivat nach einem der vorstehenden Ansprüche 8 oder 9 umfasst.

Revendications

40 1. Un peptide ayant une affinité pour les sites inflammatoires, qui comporte au moins l'une des séquences d'acides aminés suivantes:

45 LLGGPS,
 LLGGPSV,
 KEYKAKVSNKALPAPIEKTIK,
 KEYKCKVSNKALPAPIEKTIK,
 50 KTKPREQQYNSTYR et
 KTKPREQQYNSTYRVV

55 où A, C, E, G, I, K, L, N, P, Q, R, S, T, V et Y représentent les résidus aminoacides sous leur désignation conventionnelle à une lettre.

2. Un dérivé peptidique ayant une affinité pour les sites inflammatoires, qui consiste en le peptide de la revendication 1 lié à un agent de pontage chimique bi-fonctionnel.

3. Un dérivé peptidique ayant une affinité pour les sites inflammatoires selon la revendication 1, qui consiste en le peptide de la revendication 1 lié à un ligand bi-fonctionnel, dans lequel le ligand bi-fonctionnel est choisi parmi le groupe constitué par l'acide diéthylènetriamine pentaacétique, l'acide éthylènediaminetraacétique et le 1,4,7,10-téraazacyclododécane-1-aminoéthylcarbamoylméthyl-4,7,10-tris[acide (R,S)-méthylacétique].

5

4. Un dérivé peptidique ayant une affinité pour les sites inflammatoires, qui est constitué par le peptide de la revendication 2 lié à un vecteur grâce à l'agent de pontage chimique bi-fonctionnel.

10

5. Un dérivé peptidique ayant une affinité pour les sites inflammatoires selon la revendication 4, dans lequel le ligand bifonctionnel est lié à un vecteur et le ligand bi-fonctionnel est choisi parmi le groupe constitué de l'acide diéthylènetriamine pentaacétique, l'acide éthylènediaminetraacétique et le 1,4,7,10-téraazacyclododécane-1-aminoéthylcarbamoylméthyl-4,7,10-tris[acide (R,S)-méthylacétique].

15

6. Un dérivé peptidique ayant une affinité pour les sites inflammatoires selon les revendications 2, 4 ou 5, dans lequel le ligand bifonctionnel est choisi parmi le groupe constitué du sulfosuccinimidyl 4-(N-maléimideméthyl)cyclohexane-1-carboxylate, l'ester N-hydroxysuccinimide de l'acide 3-maléimidebenzoïque, le N-(ϵ -maléimidecaproyloxy)succinimide et le 4-(*p*-maléimidephényl)butyrate de succinimydyle.

20

7. Un dérivé peptidique ayant une affinité pour les sites inflammatoires selon les revendications 4 ou 5, dans lequel le vecteur est la polylysine ou la chitine désacylée.

8. Un dérivé peptidique marqué par un métal radioactif, qui est constitué du dérivé peptidique selon la revendication 3 ou 5, lié à un ion métallique radioactif.

25

9. Un dérivé peptidique marqué par un métal radioactif selon la revendication 8, dans lequel l'ion métallique radioactif est le technétium-99m ou l'indium-111.

10. Un composé pour le diagnostic comprenant le dérivé peptidique marqué par un métal radioactif selon la revendication 8 ou 9.

30

35

40

45

50

55

FIG. 1 / 1

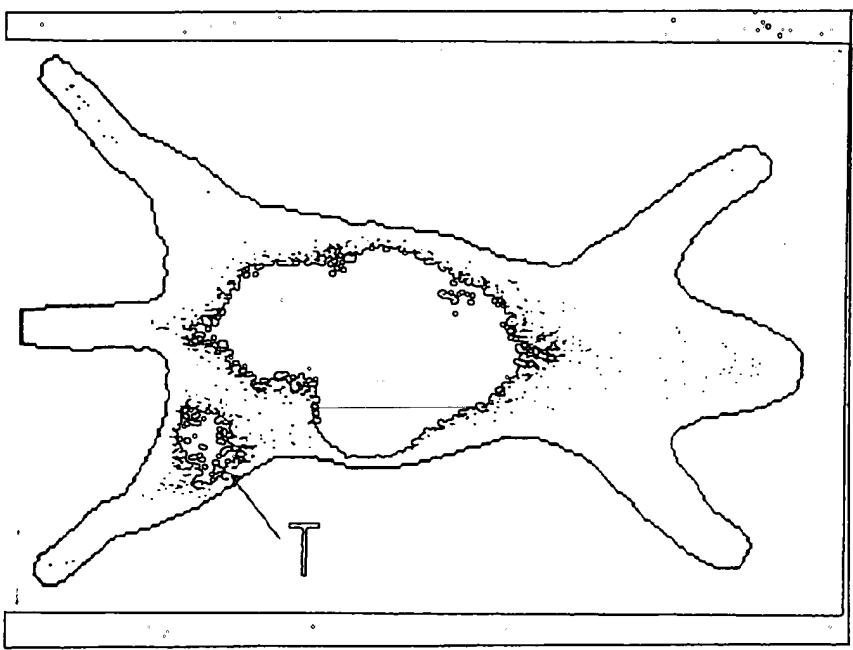
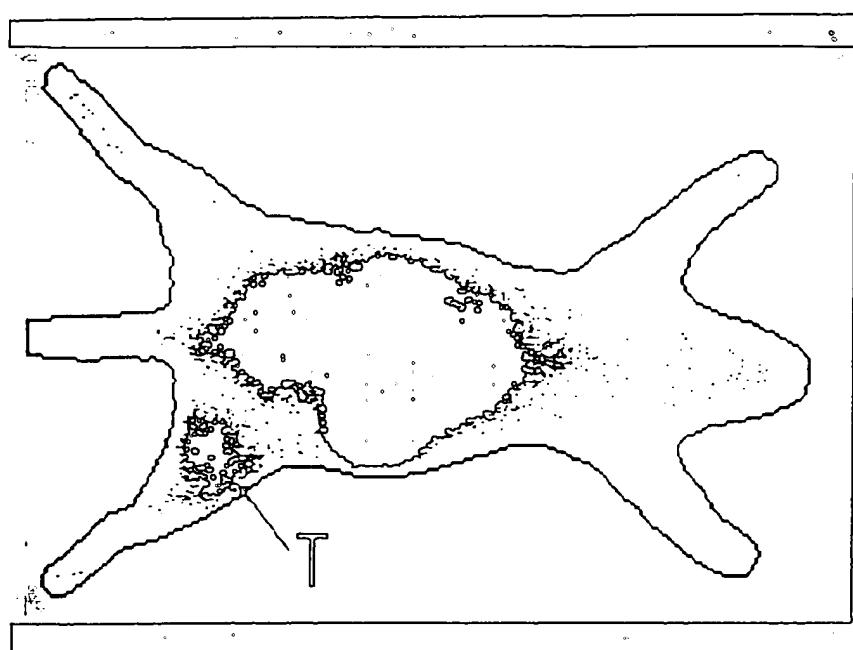


Fig. 1 / 2

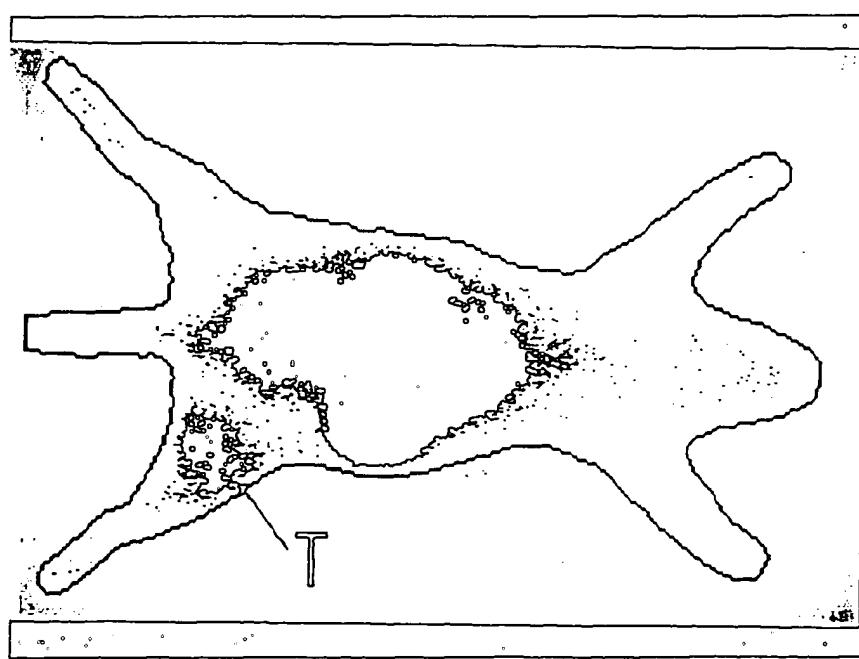


FIG. 2 /1

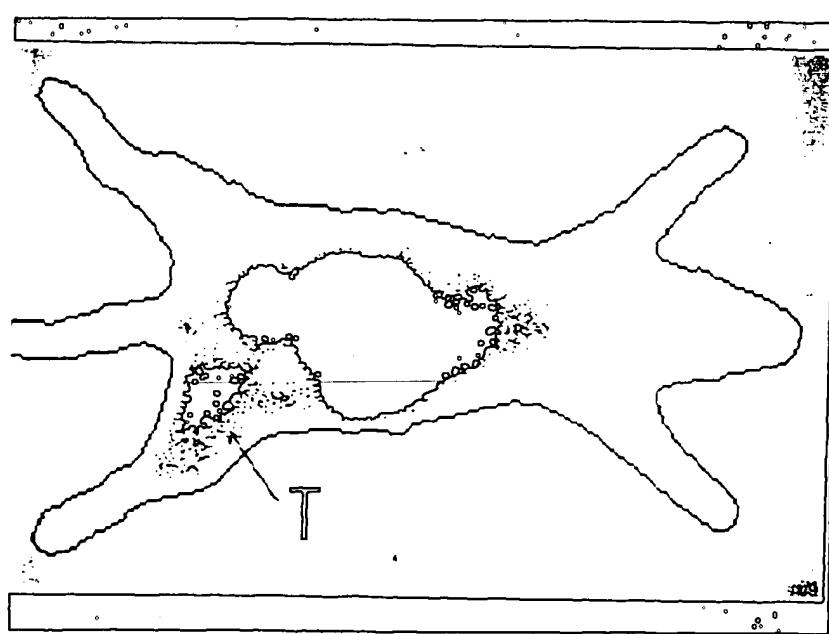
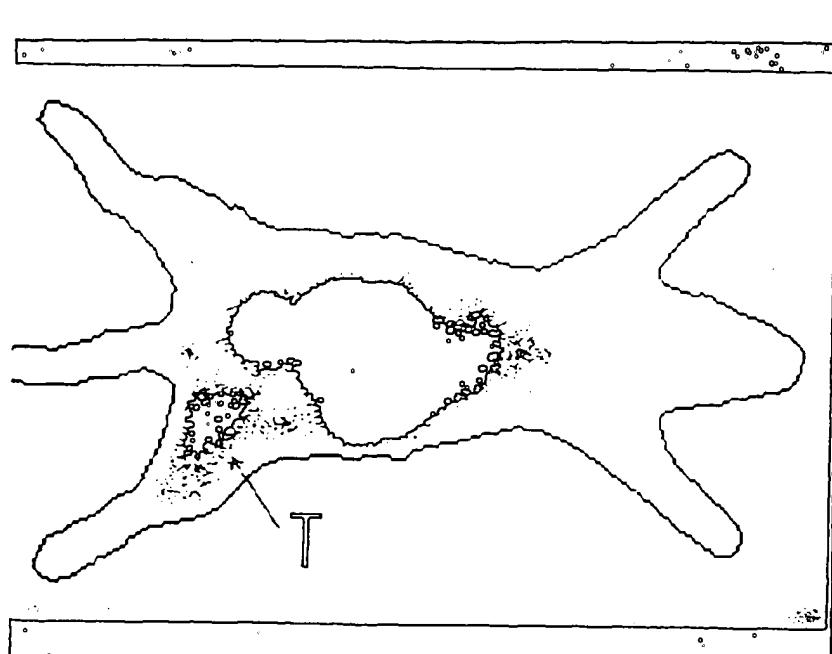


FIG. 2/2

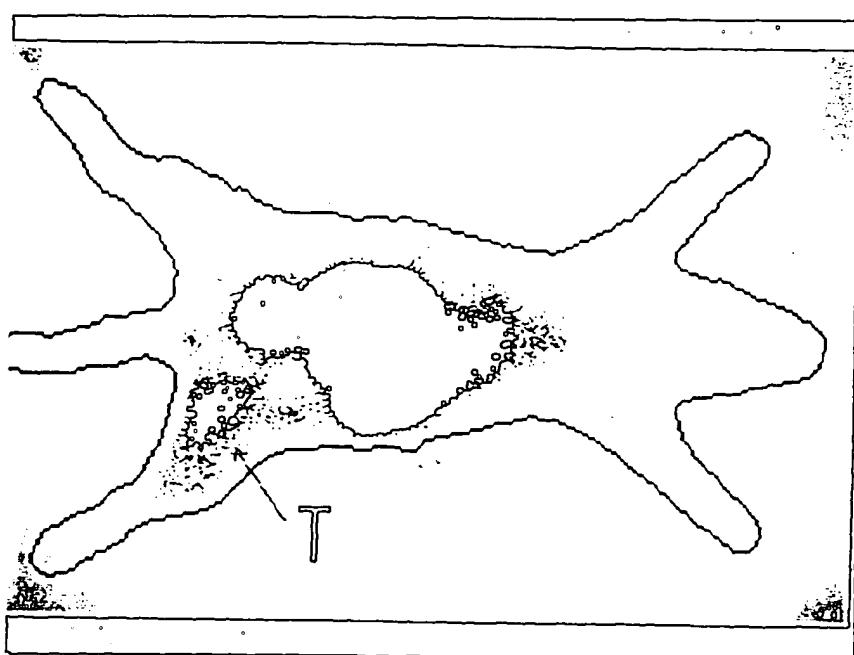


FIG. 3/1

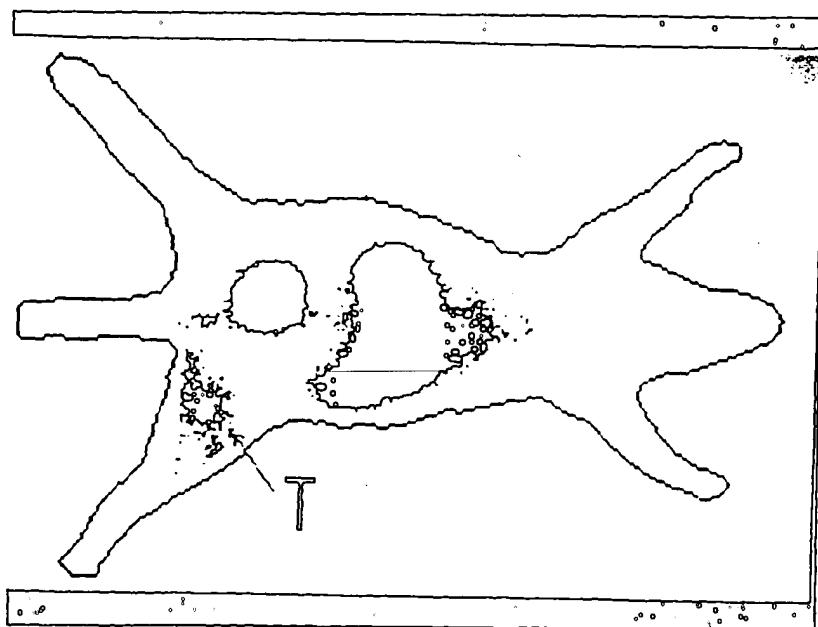
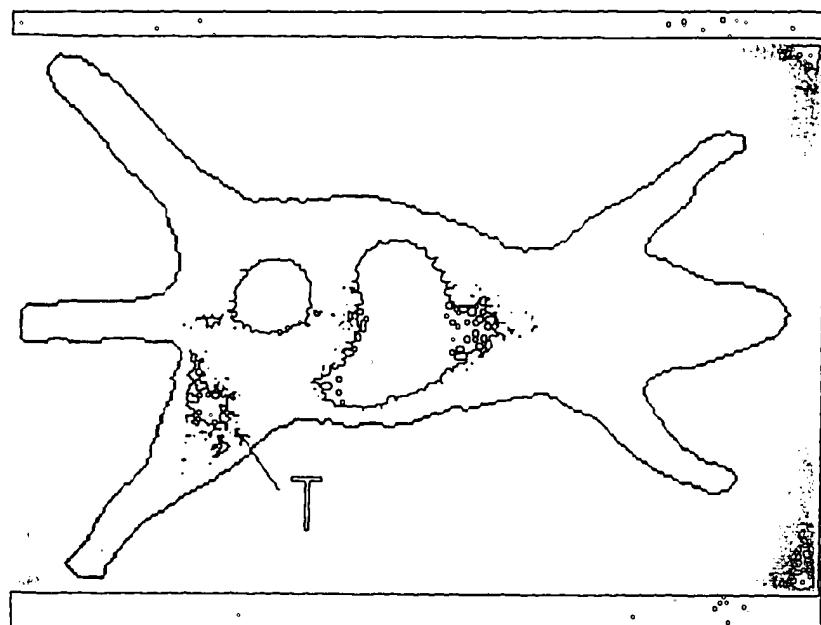


FIG. 3/2

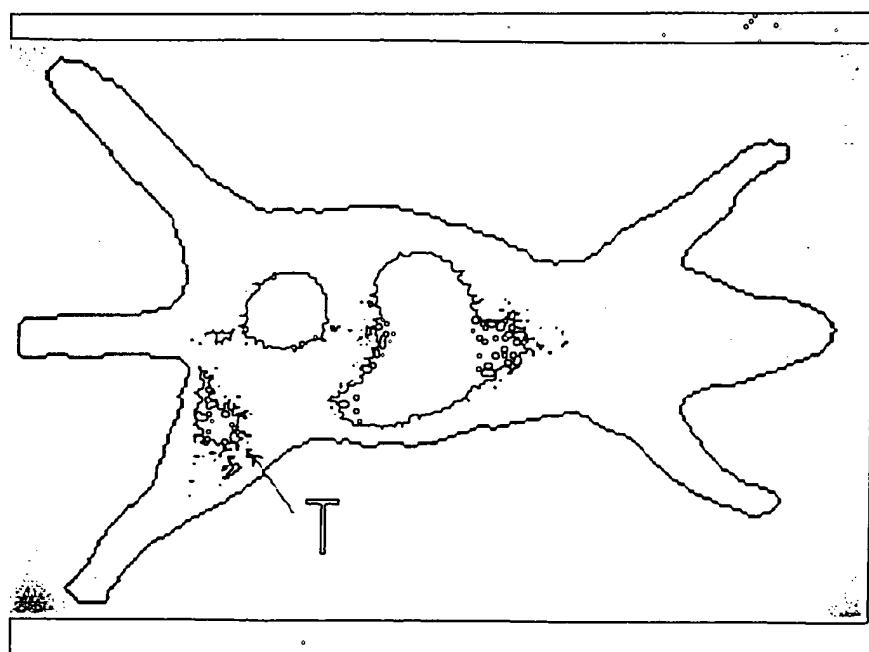


FIG. 4/1

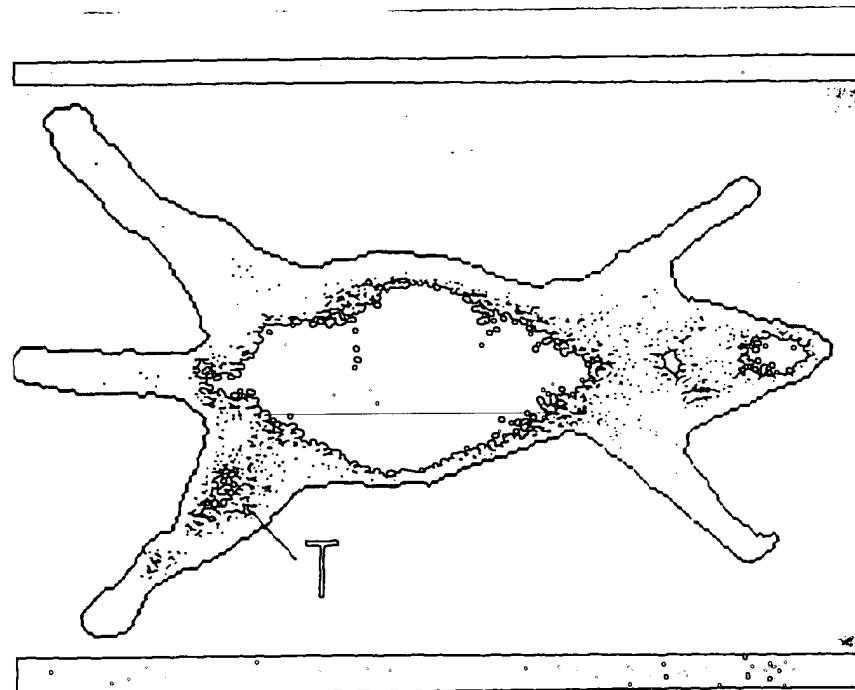
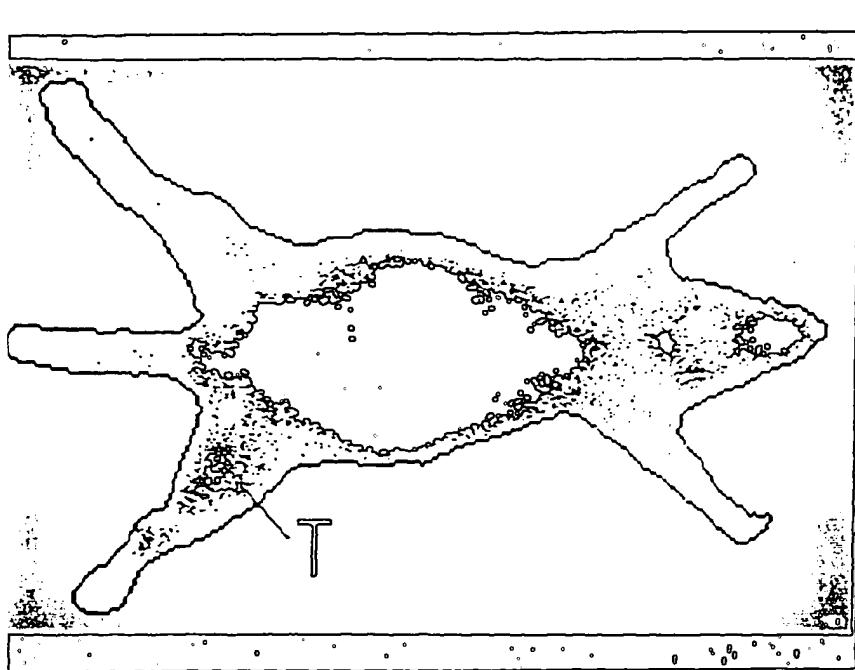


Fig. 4/2

